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Phospholipase B in the Brains and Meninges of Nonsensitized and Sensitized Rats After Challenge with *Angiostrongylus cantonensis*

ATHOS OTTOLENGHI,[1]* NORMAN F. WEATHERLY,[2] AND JOHN E. LARSH, JR.[2]

Department of Pharmacology, Duke University Medical Center, Durham, North Carolina 27710,[1] and Department of Parasitology and Laboratory Practice, University of North Carolina, Chapel Hill, North Carolina 27514[2]

After a primary infection with 100 *Angiostrongylus cantonensis* larvae, infected rats showed elevated phospholipase B activity in meningeal and brain homogenates beginning with the first week and continuing through the first month of infection. The rise in phospholipase B values through the first 4 weeks, with a prolonged peak spanning days 30 to 31, coincided with the invasion and maturation of the parasites in the brain, and the ensuing sharp decline in phospholipase B levels, shown by the readings on day 45, coincided in turn with the known migration of the worms from the brain to the lungs, which begins about 5 weeks after infection. In the meninges, the pattern of enzyme elevation was generally similar to that in the brain samples except that the highest activity was seen earlier at days 8 to 9, followed by a gradual decline by days 30 to 31 and a sharper drop by day 45. Rats challenged with 100 larvae 53 days after the primary infection exhibited an almost immediate rise of phospholipase B activity in both the brain and meninges; the peaks of activity occurred at day 1 for the meninges and day 25 for the brain, and levels above control values were still present at day 50. Comparison of the total enzymatic content of the cerebral tissue and meninges revealed that a remarkably high proportion of the phospholipase B activity was contained in the meninges. The inference that elevated levels of this enzyme in the cerebral tissue of *A. cantonensis*-infected rats are due to inflammatory reactions within the meningeal envelopes was confirmed by histochemical demonstration of specific sites of enzymatic activity limited to the meninges. It is of interest that 80% of the cells positive for the enzyme were clearly identifiable as eosinophils since an association of bone marrow eosinophilia and high phospholipase B levels in rats infected with *A. cantonensis* was shown in our earlier study of rats infected with this parasite.

A previous report from our laboratories (18) has shown that rat tissues harboring *Angiostrongylus cantonensis* exhibit abnormally high levels of phospholipase B (EC 3.1.1.5) in the organs invaded by the parasite, including the brain and lungs. The elevated enzymatic activity of brain homogenates is a point of particular interest because the brain as a whole contains normally only negligible amounts of phospholipase B and a correspondingly very small number of eosinophilic leukocytes, which, in rats, are the carrier and principal source of the enzyme in all tissues studied thus far, i.e., intestine, lung, spleen, uterus, bone marrow, and peritoneal fluid (13-15).

The present study was undertaken to determine the distribution of the enzyme in the cerebral tissues of *A. cantonensis*-infected rats and to verify the inferred dependence of its accumulation upon the presence of eosinophilic infiltrates in the affected sites.

MATERIALS AND METHODS

Rats. Male albino rats of the Charles River CD_1 strain, 75 to 100 days old, were used in this study. The rats were kept in wire cages with mesh floors and were housed in temperature-, humidity-, and light cycle (10 h of light, 14 h of darkness)-controlled rooms. Water and commercial rat pellet feed were given ad libitum.

Parasite and method of infection. *A. cantonensis* infecting larvae were obtained from snails infected and maintained by the methods of Richards and Merritt (19). The snail tissue was then digested in a 1% pepsin-1% HCl solution for 1 h at 37°C. After light centrifugation, the sediment was suspended in a gelatin-broth medium, and the desired number of larvae was introduced in the retropharynx of each rat with a blunt 18-gauge needle and syringe. To determine the number of adult worms present after infection, lungs and heart were removed from the animals, washed in saline, and teased in petri dishes for counting of the isolated worms.

Tissue preparation. The animals were anesthetized with ether and killed by decapitation. The parietal and occipital portions of the cranium were exposed

by a sagittal incision of the skin; sharp-pointed scissors were inserted in the foramen, and the occipital and temporal bones were cut on both sides along their borders with the parietal and frontal bones. The two lateral cuts were joined by a transverse cut at the level of the olfactory bulb, thereby dissecting a bone plate extending from the frontal bone to the transverse midline of the occipital bone. This portion was removed and blotted dry of blood, and the attached meninges were carefully scraped off the bone with the aid of fine-pointed tweezers beginning at the occipital fossa and proceeding to the anterior limit of the bone plate. After inspection and removal of small bone fragments and traces of blood, the small tissue sample (20 to 30 mg) was weighed on a torsion balance and homogenized in 2 ml of cold buffer (12.5% glycerol–5 $\times 10^{-3}$ M $MgCl_2$–2 $\times 10^{-3}$ M ethylenediaminetetraacetate [EDTA]–0.10 M potassium phosphate [pH 6.6]) with a hand-operated glass-glass homogenizer.

The forebrain, midbrain, pons, medulla, and cerebellum were removed by severing the connections of the olfactory and optic nerves and the nervous trunks of the base, and the tissue sample, after weighing, was minced with scissors and homogenized in 20 ml of ice-cold buffer with a Teflon-glass homogenizer.

The meningeal and brain homogenates were cleared separately by centrifugation at 600 $\times$ *g* for 10 min, and samples of the supernatants (0.6 ml) were used for the phospholipase B tests. In a few instances, fragments of the pia mater adherent to the cerebral surface were carefully peeled off the underlying nervous tissue and processed as for the dura mater samples.

Phospholipase B test. The procedure for the preparation of substrate and titration of the activity has been fully described in preceding communications (11, 13). Briefly, appropriate samples of the tissue homogenates, usually 0.6 ml, were pipetted into 15-ml centrifuge tubes, to which was added 20 μl of 0.1% trypsin. The samples were incubated at 37°C for 4 min before addition of 0.3 ml of 2 $\times 10^{-2}$ M lysolecithin prewarmed at 37°C. Incubation was continued for 60 to 90 min or until formation of cloudy precipitate (in the more active samples), and the reaction was stopped by addition of 0.1 ml of 2 N H_2SO_4. To the tubes were added, in succession, 1.0 ml of isopropyl alcohol and 0.4 ml of water. The samples were mixed and extracted with 2.0 ml of heptane. The extracted fatty acids were then titrated with 0.01 N NaOH under nitrogen by the procedure of Dole (6).

Histochemical localization of phospholipase B activity. The general procedure has been described in detail in a previous report (17). For the present study, the parietal meninges and cerebral tissue were first dissected as described above. The meninges were folded to form a small but relatively compact fragment of tissue suitable for the preparation of frozen sections. The brain was cut at the anteroposterior and transverse midlines into four parts. These samples were frozen and embedded in Tissue Tek (Ames Co., Elkhart, Ind.), and 5 to 10 sections of 6 to 10 μm thickness were cut from each sample in a cryostat. After brief fixation (10 min) in cold calcium-Formalin, the sections were transferred to an incubation medium at 37°C containing lysolecithin and cobalt acetate (0.22 $\times 10^{-2}$ M and 4 $\times 10^{-2}$ M, respectively) in 0.1 M tris(hydroxymethyl)aminomethane (Tris)-acetate buffer (pH 6.6). Sections were withdrawn at different times (160 to 180 min), rinsed free of the excess cobalt, and stained in dilute ammonium sulfide to show the cobalt precipitate formed upon liberation of the fatty acids by enzymatic hydrolysis. Reactive sites, appearing as brown-black spots over a pale yellow background, could be further differentiated from the surrounding tissue by counterstaining the cell nuclei with nuclear fast red. At this point, some of the meningeal sections were dried in air and mounted in Permount to prepare slides; others were further processed as follows to establish the identity of the reacting cells. Sections stained with sulfide and nuclear fast red were first examined with a microscope equipped with a projection attachment, and an accurate drawing of the microscopic image was delineated on paper, marking the location and distribution of the reactive cells. The sections were then incubated overnight at room temperature in 80% ethanol containing 1% HCl. This treatment removed practically all the nuclear stain and the major portion of the cobalt sulfide precipitate. The sections were then rinsed in water and incubated for 20 min at 60°C in a modified Leishman stain (1), followed by differentiation for 10 to 15 min in acidic methanol to enhance visualization of the eosinophilic leukocytes. Wet- or Permount-mounted slides were then reexamined with the projecting microscope, and matching of the eosin-positive and phospholipase-positive cells was carried out by superimposition of the microscopic image upon the outline of the first drawing.

In the course of a more detailed study of the reactive sites in brain tissue, the whole brain was cut in 10-μm sections, and 2 successive sections of each 20 were processed for incubation with lysolecithin for 60 and 180 min, followed by staining with sulfide and counterstaining with nuclear fast red. The sections were then examined and scored for the presence and histological distribution of phospholipase B-positive cells and areas.

RESULTS

Tests after primary and challenging infection with *A. cantonensis*. Each of 23 rats in group A and 11 in group B was infected with 100 larvae of *A. cantonensis*. Two of the rats in group A harbored an average of 68.5 adult worms in the lungs and heart 56 days after infection, proving the viability of the larvae in the inoculum used for the primary infection. Also in group A, five rats were killed 16 days after infection for histochemical studies. The remaining 16 rats in this group were killed for determinations of phospholipase B activity; 2 rats were killed on days 8 and 9, 15 and 16, and 30 and 31. The last four rats were killed on day 45. The 11 rats of group B were used to test the enzyme response in partially immune animals after the challenging infection. These rats were treated orally with thiabendazole (200 mg/kg) on days 14 to 18 of the primary infection in an attempt to kill the

adult worms. The presence of an average of 3.5 worms in two of these rats 34 days after treatment provided an indication of the effectiveness of this drug. Seven of the remaining nine rats in this group were challenged with 100 larvae 35 days after treatment, and three were killed at 1 day, two at 25 days, and two at 56 days after challenge to determine the phospholipase B activity in their tissues. The larvae in the challenge inoculum were viable as shown by the average recovery of 59.5 worms from two additional previously uninfected rats at 56 days after infection. Also, the fact that an average of only 32.5 worms was recovered at this time from the remaining two treated and challenged rats proved that the primary infection had produced a significant degree of immunity ($P = 0.001$).

In addition to the rats in groups A and B, it was necessary to include control (uninfected) rats for comparison of phospholipase B activity. Two uninfected rats were killed and tested for phospholipase activity on days 8 and 15, and one was killed and tested on days 30, 57, 81, and 112 after the primary infection had been given to the experimental rats.

Tables 1 and 2 summarize the changes in phospholipase B activity of the brain and meninges, respectively, referred to unit (wet weight) of tissue examined. In Table 3, the values, derived from the same experiments, represent the actual total phospholipase B activity present in the whole brain and in the sample of meningeal tissue tested. In all tables, the data for two consecutive days (8 and 9, 15 and 16, and 30 and 31) were pooled since no systemic difference was discernible in readings made within a 24-h interval.

The photomicrographs in Fig. 1 and 2 show the localization of the phospholipase B in discrete cellular sites in the meningeal envelopes isolated from the parietal bone (Fig. 1) and in the narrow meningeal fold overlying the cerebral tissue (Fig. 2).

Two additional groups of rats exposed to primary infection with 100 larvae were studied. The first group consisted of 12 animals tested in groups of four per day on days 2, 4, 6, and 8 with the intent of detailing the time course of initial rise in phospholipase B activity. The experimental values for both meningeal and brain homogenates of animals examined on days 2 through 6 did not differ from those of uninfected controls; readings clearly above the normal range were found in animals killed on day 8, consonant with the results of the previous series of tests (data not given). The five animals of the second group were killed on days 13, 16, 19, 30, and 45, and serial sections of the entire brain were incubated with lysolecithin to show the distribution of the phospholipase B activity within the cerebral tissue. In brief, phospholipase B-positive cells were present in large numbers in the meninges of rats killed within the interval from day 13 to 30 but were not seen in the animal examined on day 45. With few exceptions, these cells, similar in all aspects to those shown in Fig. 2a and b, occurred as single cellular elements located in the meninges covering the cerebral surface or in the perivascular space of the interlobar areas. Multicellular aggregates showing diffusion of the phospholipase B activity to the surrounding meningeal tissue were seen only in a limited number of histological sections of the cerebellum of the animal examined on day 30. The number and the distribution of the cells containing the enzyme bore no apparent relation to the location of live worms in the cerebral surface or in the blood vessels. In contrast to the relative abundance of reactive sites in the meninges, foci of phospholipase B activity could not be detected

TABLE 1. *Phospholipase B levels in the brains of A. cantonensis-infected rats*

Rats	Activity[a] at following day after primary infection:				Activity[a] at following day after reinfection:		
	8–9	15–16	30–31	45	1	25	56
Infected	12.1	21.6	39.6	4.7	14.2	20.9	8.6
	9.6	14.4	38.9	13.6	1.8	35.5	9.1
	9.1	23.0	50.8	14.5	30.4		
	7.8	3.3	67.8	14.8			
Mean (SE)	9.6 (0.9)	15.6 (4.5)	49.3 (6.8)	11.9 (2.4)	15.5 (8.3)	28.2 (7.3)	8.8 (0.2)
Control	3.2	0.2	0.25		5.6	3.9	1.7
	2.7	0.9					
Mean (SE)	2.3 (0.7)[b]						

[a] Activity expressed as micromoles of lysolecithin hydrolyzed per gram of tissue per hour.
[b] Mean of combined control rats.

TABLE 2. *Phospholipase B levels in the meninges of A. cantonensis-infected rats*

Rats	Activity[a] at following day after primary infection:				Activity[a] at following day after reinfection:		
	8–9	15–16	30–31	45	1	25	56
Infected	503	606	267	58	303	21	12
	198	286	153	71	38	58	22
	735	410	383	89	238		
	139	17	53	98			
Mean (SE)	394 (139)	330 (123)	214 (71)	79 (9)	193 (80)	40 (18)	17 (5)
Control	67	15	11		18	26	10
	23	17					
Mean (SE)	23 (6)[b]						

[a] Activity expressed as micromoles of lysolecithin hydrolyzed per gram of tissue per hour.
[b] Mean of combined control rats.

TABLE 3. *Phospholipase B content of brain and meninges in A. cantonensis-infected rats*

Rats	Activity[a] at following day after infection:									
	8–9		15–16		30–31		45		56 + 1[b]	
	B	M	B	M	B	M	B	M	B	M
Infected	18.6	8.0	33.0	16.4	79.2	11.2	7.0	0.7	22.4	7.9
	15.8	5.0	23.7	11.4	66.9	6.4	21.3	2.3	45.0	9.5
	13.7	16.2	36.1	13.5	76.2	18.4	20.0	2.7	**[c]	**
	11.7	2.5	**[c]	**	94.2	2.5	20.3	3.5		
Mean	15.0	7.9	30.9	13.8	79.1	9.6	17.1	2.3	33.7	8.7
Control[d]	3.0	0.6								

[a] Micromoles of lysolecithin hydrolyzed per hour in homogenates of brain (B) and meninges (M).
[b] Reinfected rats.
[c] **, Nonresponding animal omitted.
[d] Mean of eight animals.

within the nerve tissue of the brain, even in areas immediately adjacent to cast skins and parasite tracks.

DISCUSSION

In appraising the significance of the data reported here, it must be pointed out that the values relative to the uninfected control rats for both brain and meningeal homogenates are actually computed from titration readings often only marginally above the reagent blanks for samples incubated in the absence of substrate. Hence, it can be stated that the tissues from the controls contained at most only traces of phospholipase B activity. In contrast, clearly positive titration values were present in the infected rats beginning at the first week and continuing through the first month after infection. Two exceptions were noted on days 15, 16, and 57 (Tables 1 and 2) since these animals exhibited activity within the control range. The most likely explanation for this finding is a technical failure to infect the animals in question, projecting a failure rate of 1/12, which is consonant with common laboratory practice with this experimental model.

As shown in Table 1, the data for the brain tissue reveal a rather clear pattern of rising phospholipase B values beginning on day 8 and reaching a peak at days 30 to 31 of infection. This time interval coincides with the maturation phase of the parasite in the brain, whereas the ensuing phase of migration to the lungs, beginning about the fifth week after infection, precedes the sharp decline of the phospholipase levels shown by the readings at day 45.

These results are in agreement with the findings of a previous study (18), wherein a similar pattern and an overall similar range of experimental values had been determined. The same conclusion applies to the reinfected group exhibiting an almost immediate (day 1) rise in

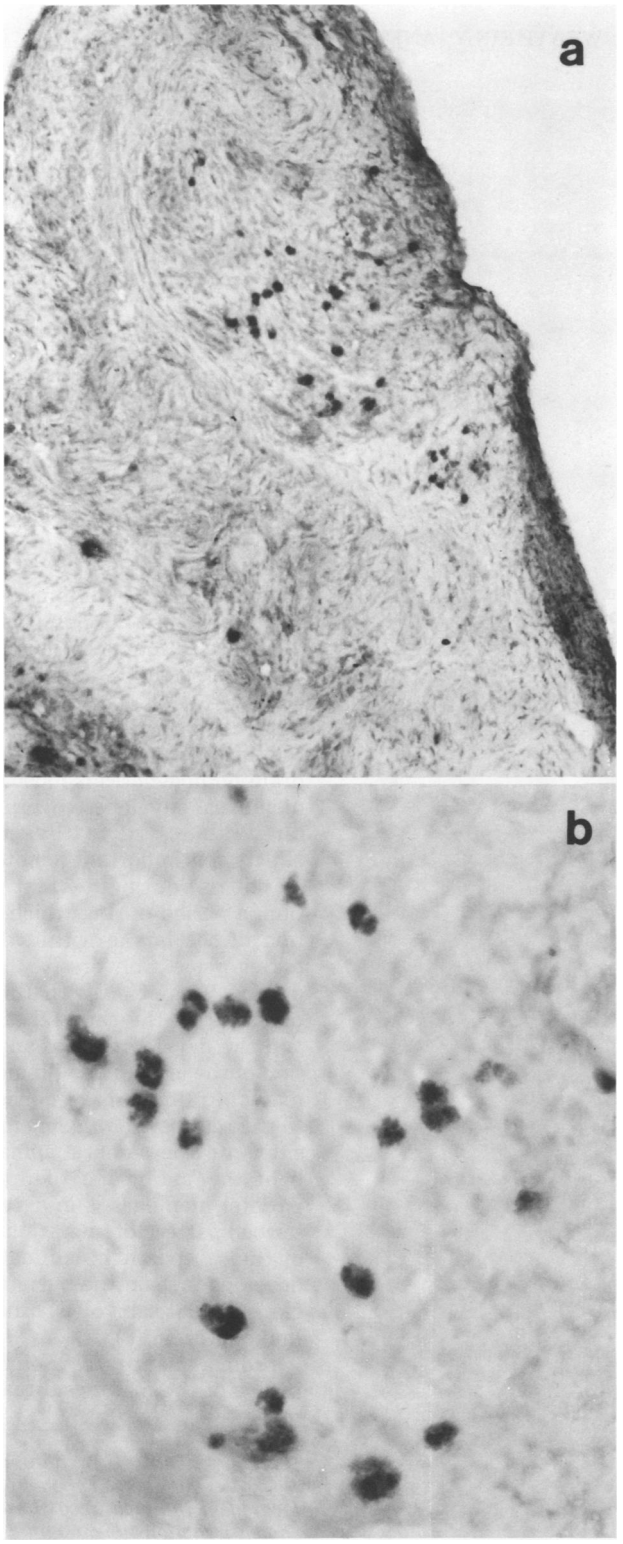
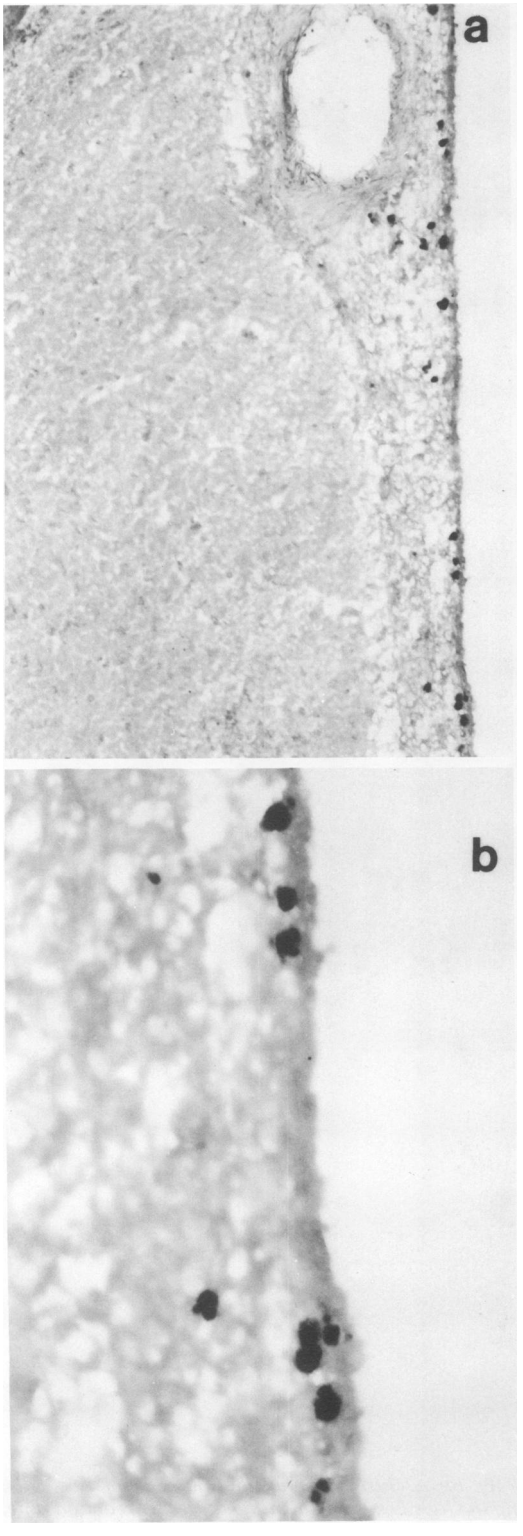


FIG. 1. *Meninges of A. cantonensis-infected rat (day 16). (a) A cluster of phospholipase B-positive cells, containing a brown-black precipitate, is shown infiltrating the lightly stained meningeal tissue (reaction time, 60 min; counterstain with nuclear fast red; ×130). (b) Intracellular localization of the precipitate around lighter nuclear areas is visible in several cells at higher magnification (×400).*



enzyme activity in two of the three rats tested.

The data of Table 2 show the occurrence of phospholipase B accumulation in the meninges during the active cerebral phase of the infection. It is apparent that enzyme levels rise to values strikingly above the range of the uninfected controls in the interval of days 8 to 31 of infection and on day 1 of reinfection. Two clear exceptions (on days 15 and 16 of the once-infected and day 1 of the reinfected rats) are for the same animals that showed no increase in the brain enzyme values (Table 1). The changes in the phospholipase B content of the meninges, as a whole, follow a time course parallel to the progress and resolution of the inflammatory process accompanying the infection; when compared with the data of Table 1, they show an earlier peak response without apparent correlation with the levels of phospholipase B activity seen in the corresponding brain homogenates.

Although it is not possible to offer a verifiable, definitive rationale for this discrepancy, the following considerations seem relevant to the conclusion to be put forth here, i.e., that the meninges are actually the site of accumulation of the enzyme and that the activity found in the brain homogenates is most likely located in the meningeal envelopes covering the cerebral matter. With regard to the time course of these effects, it is important to note that enzyme levels do not rise during the first six days after infection, a period during which molting and migration of the larvae within the nerve tissue are taking place. Conversely, the dramatic changes on day 8 coincide with the emergence of the parasites on the cerebral and cerebellar cortex and the development of an inflammatory reaction of the arachnoid villi and the adjacent dura mater (9, 10). This response has been ascribed to the release of antigenic material by the parasites into the cerebral spinal fluid, and, in this light, the finding of earlier peak responses in the parietal meninges is in agreement with the described pattern of migration of the parasites and of the attendant inflammatory reaction which develops first on the parietal and cerebellar regions and later involves other areas of the cranium and brain (8).

From a quantitative point of view, the preeminence of the meningeal reaction is emphasized

FIG. 2. *Brain of A. cantonensis-infected rat (day 16). (a) A number of phospholipase B-positive cells located in the meningeal lining of brain tissue are shown (reaction time, 60 min; counterstain with nuclear fast red; ×130). (b) The intracellular localization of the precipitate is visible at higher magnification (×400).*

in the data of Table 3, wherein the total enzymatic contents of the meningeal and brain homogenates are compared. It is apparent from these data that, in fact, the small and limited meningeal fragments (averaging 28 mg) used in these determinations had a remarkably high content of phospholipase B activity, averaging, on the aggregate, approximately one-half of the amount present in the homogenates of the whole cerebral tissue and attached meningeal envelopes examined on days 8, 9, 15, and 16.

The inference that the phospholipase B activity of the cerebral homogenates is actually localized in the meninges enveloping the organ is supported also by the findings of considerable enzymatic activity in small samples of arachnoid and pia mater carefully removed from the parietal and temporal lobes and from the fissura rhinalis of rats examined during the first 2 weeks of infection. Meningeal fragments averaging only 13 mg (wet weight) exhibited activities of 4 U/h on days 8 and 9 and of up to 12 U/h on days 15 and 16, which compare favorably with the levels seen in the larger meningeal samples (28 mg) isolated from the parietal and frontal bones (Table 3).

Morphological evidence bearing on these points was obtained by the demonstration of the specific regional and cellular localization of the phospholipase B by histochemical procedures entailing incubation of sections of meninges or brain with lysolecithin, precipitation of the liberated fatty acids with cobalt, and staining of the latter with sulfide. This method, which has been shown to be of general applicability to rat tissues (17), revealed the presence of discrete, well-localized foci of activity in both the meninges and brain. In the sections of the meninges proper (dura mater and arachnoid of the parietal area), the positive cells were present in clusters infiltrating the meningeal stroma as shown in Fig. 1a and b. At higher magnification of the same slide, it was possible to observe the special arrangement of the dark precipitate surrounding the lighter annular outline of the nuclei characteristic of leukocytes. The same cells were found in sections of the brain, where their presence was limited to the narrow meningeal layer enveloping the nervous tissue (Fig. 2a and b). Both the number of cells exhibiting positive phospholipase B reaction and the amount of precipitate accumulating at each reactive site increased with time of incubation through the first 90 min; beyond this limit, some diffusion of the precipitate to the surrounding tissue became apparent. Other slides from infected rats cut in alternate sequence to the experimental slides and incubated in the absence of lysolecithin were uniformly negative, as were sections of tissues of control (uninfected) rats.

These results were complemented by the later series of tests in animals examined during the interval from day 13 to 30 that confirmed the meningeal location of the reactive cells, which, in a few instances, were arranged in small granuloma-type formations. A negative finding of this experiment which deserves comment is the failure to show phospholipase B-positive sites within the cerebral tissue proper. This is at variance with the observations of Jindrak (9), who reported the presence of eosinophil-rich granulomas surrounding dead larvae during the period from day 12 to 20 postinfection. It is therefore somewhat surprising that none of the several hundred slides of brain examined in this study revealed the presence of reactive foci in sites other than the meninges. Although it is possible that technical factors, such as the inevitable loss of tissue during the mounting, trimming, and sectioning of the samples and the selection of 2 out of 20 successive sections for reacting with substrate, might have reduced the likelihood of detecting the rather limited number of reactive sites present in the brain, it is also to be noted that the infecting dose used in these experiments was considerably smaller (100 versus 500) than that given to the animals examined by Jindrak on days 12 to 18 of infection, a time when the eosinophilic infiltrates were most numerous and extensive.

From all the evidence reviewed above, it appears that phospholipase B activity does not accumulate within the nerve tissue to any major, significant extent in rats infected with 100 larvae; in contrast, the meninges are positive for this activity on the basis of biochemical and histochemical data which can be correlated with known phases of the infective process.

The initial tentative identification of the positive cells as eosinophilic leukocytes, drawn from the striking similarity in the number, arrangement, and distribution of phospholipase B-carrying cells and eosinophils in slides stained specifically for one or the other property, was confirmed by the more direct and conclusive evidence emerging from the experiments involving double staining with matching of the reactive cells on projections of the microscopic images. More than 80% of the cells positive for the enzyme could be clearly identified as eosinophils; conversely, 90% of the eosinophils were identified as phospholipase B-carrying cells. These are acceptable discrepancies considering the technical limitations of the method, which is dependent upon approximately equivalent reaction rates in the cells for the demonstration of

enzymatic activity and upon preservation of tinctorial characteristics for cell typing in sections previously reacted with substrate and stain.

Therefore, the combined evidence of this study confirms the association between eosinophils and phospholipase B already documented for a number of other rat tissues (13–15, 17) and warrants the conclusion that the elevated levels of phospholipase B activity demonstrable in the cerebral tissue of *A. cantonensis*-infected rats are due to infiltration of the meninges by these cells as part of the inflammatory reaction to antigenic stimuli released by the parasites.

The occurrence of such a response in a tissue normally almost devoid of the enzyme and its carrier cells is of special interest, since it provides a clear demonstration that accumulation of phospholipase B is an integral component of the tissue reaction causally and temporally related to invasion by *A. cantonensis*. The direct demonstration of eosinophils as the phospholipase B-carrying cells is in keeping with the earlier demonstration of an infection-related bone marrow eosinophilia in rats infected with this parasite (18).

As for previous studies, wherein the relation between eosinophils, phospholipase B, and helminth infection was investigated (11, 12, 16, 18), two basic questions require consideration. The first, concerning the link between eosinophil mobilization and infection, has been clarified by the work of Basten et al. (2, 3), who have shown the role of the lymphocytes in mediating the proliferating stimulus to the bone marrow and in determining the earlier and magnified response upon reintroduction of the causative agent. What is to be underlined in the present context is the immediate rise in phospholipase B in the twice-infected animals, a result which is in agreement with the amnestic-type response to be expected upon reinfection, as has already been seen with other helminths (12, 16). The second and as yet unanswered question concerns the relation between the accumulation of phospholipase B and the course of helminth infection. The association of the two processes is by now a well-documented occurrence in a variety of tissues and in different conditions of humoral and inflammatory response (11, 12, 16, 18). Since, in general, the time course of the enzyme accumulation parallels the local reaction to the invading parasites up to their expulsion or migration from the infected site, it is conceivable that phospholipase B might contribute directly or indirectly to the containment of the infection. It is noteworthy that a similar specific role has been assigned to the carrier cell, i.e., the eosinophilic leukocyte, in relation to the killing of schistosomula and resistance to *Trichinella spiralis* (4, 5, 7). In this connection, it can be added here that, in studies now in progress, accumulation of phospholipase B has been found to occur also in mice and rats infected with *Schistosoma mansoni*. Although all these considerations can be said to contribute to the plausibility of a role for the enzyme in the tissue reaction to the parasites, definition of its function must await clarification of the biochemical and cellular changes consequent to its presence in the abnormally high levels found in the infected organs.

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